Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent

Bey-Dih Chang, Mari E. Swift, Mei Shen, Jing Fang, Eugenia V. Broude, and Igor B. Roninson*

Department of Molecular Genetics, University of Illinois, 900 South Ashland Avenue, Chicago, IL 60607-7170

Communicated by Emanuel Margoliash, University of Illinois, Chicago, IL, November 9, 2001 (received for review August 31, 2001)

Treatment with chemotherapy or radiation is not invariably cytotoxic to all tumor cells. Some of the cells that survive treatment recover and resume proliferation, whereas others undergo permanent growth arrest. To understand the nature of treatment-induced terminal growth arrest, colon carcinoma cells were exposed to doxorubicin, and surviving cells were separated into proliferating and growth-arrested populations. Only growth-arrested cells displayed phenotypic markers of cell senescence and failed to form colonies. Gene expression was compared between senescent and proliferating fractions of drug-treated cells by using cDNA microarray hybridization and reverse transcription–PCR. Drug-induced senescence was associated with inhibition of genes involved in cell proliferation and with coinduction of multiple intracellular and secreted growth inhibitors. Several tumor suppressors and other genes that are down-regulated in carcinogenesis were up-regulated in senescent tumor cells. Induction of most growth inhibitors was delayed but not abolished in cells with homozygous knockout of p53, in agreement with only limited p53 dependence of drug-induced terminal growth arrest. On the other hand, senescent cells overexpressed secreted proteins with antiapoptotic, mitogenic, and angiogenic activities, suggesting that drug-induced senescence is associated with paracrine tumor-promoting effects. About one-third of the genes up-regulated in senescent cells and almost all of the down-regulated genes showed decreased or delayed changes in $p21^{Waf1/Cip1/Sdi1}$-deficient cells, indicating that p21 is a major mediator of the effects of p53 on gene expression. Elucidation of molecular changes in tumor cells that undergo drug-induced senescence suggests potential strategies for diagnostics and therapeutic modulation of this antiproliferative response in cancer treatment.

Irreversible proliferation arrest in tumor cells treated with anticancer agents may result from cell death or permanent growth arrest. The mechanism of damage-induced cell death is a subject of active investigation, but little is known about the determinants of terminal growth arrest in tumor cells. We have previously shown that exposure of different tumor cell lines to various anticancer agents *in vitro* and *in vivo* induces long-term growth arrest with phenotypic features of cell senescence, such as cell enlargement, increased adhesion and granularity, and senescence-associated β-galactosidase activity (SA-β-gal) (1). Tumor cells can also be induced to undergo senescence through ectopic expression of tumor suppressors or oncogene inhibition. For example, inhibition of papillomavirus oncoproteins E6 and E7 in cervical carcinoma cell lines was found to induce senescence-like growth arrest in almost 100% of cells (2). Activation of the senescence program in tumor cells seems, therefore, to be a feasible biological approach to cancer therapy.

In normal cells, senescence may develop as a result of telomere shortening after multiple cell divisions (3) (replicative senescence) or may be triggered by DNA damage (4, 5) or RAS mutations (6) (accelerated senescence). Growth arrest of normal senescent cells is mediated by the activation of p53, which arrests the cell cycle primarily through the induction of $p21^{Waf1/Cip1/Sdi1}$, a pleiotropic inhibitor of cyclin-dependent kinases (CDKs). p21 induction in senescent cells is transient and is followed by stable activation of another CDK inhibitor, $p16^{Ink4A}$. p16 is believed to be responsible for the maintenance of growth arrest in senescent cells after the shutoff of p21 (5, 7). The p53–p21–p16 cascade, however, may be insufficient to account for accelerated senescence in tumor cells. p53 and p16 (but not p21) are two of the most frequently inactivated genes in human cancer. Nevertheless, p16-deficient tumor cell lines readily undergo senescence-like growth arrest in response to DNA damage (1). Inhibition or knockout of p53 or p21 diminishes but does not abolish the senescence response in tumor cells treated with different agents (8). These findings suggest that p53 and p21 are positive regulators but not the sole determinants of treatment-induced senescence in tumor cells. In the present study, we have identified genes associated with growth arrest and other aspects of treatment-induced senescence in tumor cells and determined the roles of p53 and p21 in the regulation of senescence-associated genes.

Materials and Methods

Cellular Assays. HCT116 wild-type, p21−/− (clone 80S4), and p53−/− (clone 379.2) cell lines (a gift of B. Vogelstein, Johns Hopkins University, Baltimore) were grown in DMEM with 10% FC2 serum. For fluorescence-activated cell sorter (FACS) analysis of cell division, HCT116 cells were labeled with PKH2 and plated at 5×10^6 cells per 15-cm plate. After a 24-hr incubation with 200 nM doxorubicin, cells were allowed to recover in drug-free media for up to 10 days. PKH2 labeling, FACS analysis, and cell sorting were carried out as described (1, 8). Sorted fractions of $PKH2^{hi}$ and $PKH2^{lo}$ cells (90–95% purity) were analyzed for DNA content by using propidium iodide staining and FACS analysis (9), stained for SA-β-gal activity as described by Dimri *et al.* (10), and tested for clonogenicity by plating 2,000–10,000 sorted cells per 10-cm plate.

Gene Expression Analysis. Poly(A)+ RNA and protein extracts were prepared from $PKH2^{lo}$ and $PKH2^{hi}$ cell populations and separated in different experiments 6, 9, or 10 days after release from doxorubicin. Synthesis of fluorescent cDNA probes, hybridization with the human UniGEM V 2.0 cDNA microarray, and signal analysis were conducted by IncyteGenomics (St. Louis), as described by the manufacturer's web site (www.incyte.com). Of >9,000 sequence-verified genes and expressed sequence tags present in the UniGEM V 2.0 microarray, 82% gave measurable hybridization signals with both probes. Changes in gene expression were verified by semiquantitative reverse transcription (RT)-PCR, essentially as described (11), using β-actin as an internal normalization standard. Sequences of RT-PCR primers and PCR conditions will be provided on request. RT-PCR analysis was carried out by using two pairs of proliferating- and senescent-cell RNA preparations isolated in

Abbreviations: FACS, fluorescence-activated cell sorter; RT, reverse transcription; SA-β-gal, senescence-associated β-galactosidase; ECM, extracellular matrix; TGF, transforming growth factor.

*To whom reprint requests should be addressed. E-mail: roninson@uic.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

independent experiments, with the same results. For a subset of the genes, the assays were reproduced with the same pair of RNA samples. Immunoblotting assays were carried out at least twice (with the same results), using the following primary Abs: mouse mAbs against β-actin (Sigma), p53 and p21 (Oncogene Research, San Diego), Maspin (PharMingen), keratin 18 (NeoMarkers, Fremont , CA), cyclin D1 (Santa Cruz Biotechnology), and rabbit polyclonal Abs against ATF-3 (Santa Cruz Biotechnology), Mad-2 (Babco, Richmond, CA), and EPLIN (*e*pithelial *p*rotein *l*ost *i*n *n*eoplasm; a gift of D. Chang, Univ. California, Los Angeles). Bands were detected by using horseradish peroxidase-labeled secondary Abs and enhanced chemiluminescence detection kit (Amersham Pharmacia).

Results and Discussion

Separation and Characterization of Proliferating and Senescent Populations of HCT116 Carcinoma Cells That Survive Doxorubicin Treatment. We have analyzed chemotherapy-induced accelerated senescence in human HCT116 colon carcinoma cell line, which is wild type for p53 but does not express p16 (12). HCT116 cells were treated for 24 h with 200 nM doxorubicin. This widely used anticancer drug produces DNA damage by stabilizing a cleavable intermediate complex formed by topoisomerase II in the process of DNA segregation. Before treatment, cells were labeled with a lipophilic fluorophore PKH2, which stably incorporates into the plasma membrane and distributes evenly between daughter cells, resulting in gradual decrease in PKH2 fluorescence during consequent cell divisions (13). Changes in PKH2 fluorescence were monitored by FACS on different days after doxorubicin treatment. Cells that died after drug treatment were excluded from this analysis based on their staining with membrane-impermeable dye propidium iodide (PI). Almost all PI-negative cells remained growth-arrested ($PKH2^{hi}$) for the first 2–3 days after doxorubicin treatment, but a proliferating cell population ($PKH2^{lo}$) emerged starting from day 4 (Fig. 1*A*). A substantial fraction of cells, however, remained $PKH2^{hi}$ and did not decrease their fluorescence, indicating that these cells did not divide even once after release from the drug. Six to ten days after doxorubicin treatment, the surviving cells were separated by FACS into $PKH2^{hi}$ and $PKH2^{lo}$ fractions. DNA content analysis showed that most of $PKH2^{hi}$ cells remained in G_2 (Fig. 1*B*), the phase where most of the cells had been originally arrested by doxorubicin through its effect on topoisomerase II. As shown in Fig. 1*C*, $PKH2^{hi}$ cells were greatly enlarged and stained positively for SA-β-gal, indicating their senescent phenotype. In contrast, $PKH2^{lo}$ cells retained normal size and remained negative for SA-β-gal. The ability to form colonies was essentially confined to the $PKH2^{lo}$ fraction (Fig. 1*D*), indicating that the senescent $PKH2^{hi}$ cells have lost their proliferative capacity.

Analysis of Changes in Gene Expression Associated with Drug-Induced Senescence: Inhibition of Genes Involved in Cell Proliferation. Fluorescent cDNA probes were prepared from RNA of senescent ($PKH2^{hi}$) and proliferating ($PKH2^{lo}$) cell populations and used for differential hybridization with UniGEM V 2.0 human cDNA microarray, containing >9, 000 genes. Lists of genes identified by this hybridization as down-regulated or up-regulated in the senescent relative to proliferating cells (with balanced differential expression of 2.0 or higher) are provided in Tables 1 and 2, which are published as supporting information on the PNAS web site, www.pnas.org. RT-PCR analysis (Fig. 2*A*) was carried out for 74 individual genes and confirmed qualitative changes in gene expression for 26/29 down-regulated and 37/45 up-regulated genes. In most cases, differences in gene expression revealed by RT-PCR were much higher than the values indicated by cDNA microarray hybridization. Changes in the expression of seven genes were also confirmed at the protein level by immunoblotting (Fig. 3*A*).

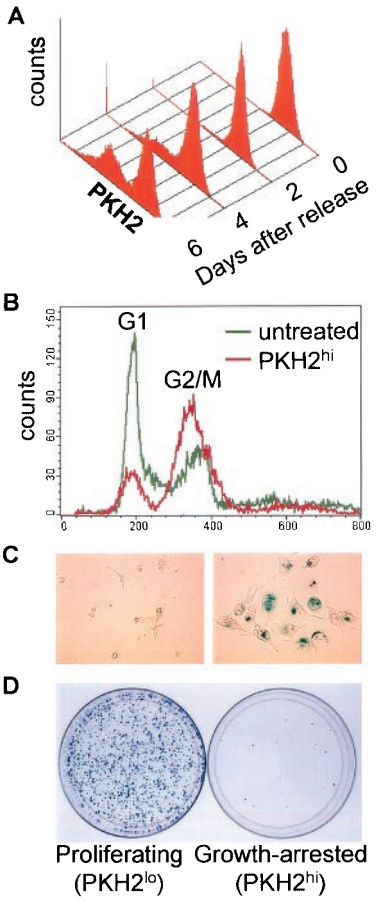

Fig. 1. Separation and analysis of proliferating and growth-arrested fractions of doxorubicin-treated HCT116 cells. (*A*) FACS profiles of PKH2 fluorescence of HCT116 cells on the indicated days after release from doxorubicin. $PKH2^{lo}$ population of proliferating cells appears on day 4 and separates from the $PKH2^{hi}$ (growth-arrested) population by day 6. (*B*) FACS analysis of DNA content of exponentially growing HCT116 cells (green) and of $PKH2^{hi}$ population isolated 9 days after drug treatment (red). (*C*) SA-β-gal staining of $PKH2^{hi}$ (*Right*) and $PKH2^{lo}$ (*Left*) populations, separated 6 days after release from the drug. Both *Left* and *Right* are photographed at the ×200. (*D*) Colony formation by $PKH2^{hi}$ and $PKH2^{lo}$ populations, separated 9 days after drug treatment and plated at 10,000 live (propidium iodide-negative) cells per 10-cm plate.

More than one-half of 68 genes and expressed sequence tags down-regulated in senescent cells are known to play a role in cell cycle progression. Of these genes, 25 are involved in different stages of mitosis or DNA segregation (e.g., CDC2, Ki-67, MAD2, and topoisomerase IIα), 11 genes function in DNA replication and chromatin assembly (e.g., ribonucleotide reductase M1, thymidylate kinase, and replication protein A3), and 4 genes are involved in DNA repair (e.g., *HEX1* and *FEN1*). Down-regulation of genes involved in cell proliferation correlates with the growth-arrested state of senescent cells and demonstrates the biological relevance of gene expression profiling in our system.

Induction of Multiple Growth-Inhibitory Genes and Apparent Reversal of Neoplastic Transformation in Senescent Cells. Senescent HCT116 cells were found to up-regulate multiple genes with documented growth-inhibitory activity, providing an ample explanation for the maintenance of doxorubicin-induced cell cycle arrest in the absence of p16. One of the up-regulated genes is p21 (Fig. 2*A*). Analysis of p21 and p53 protein induction by doxorubicin in

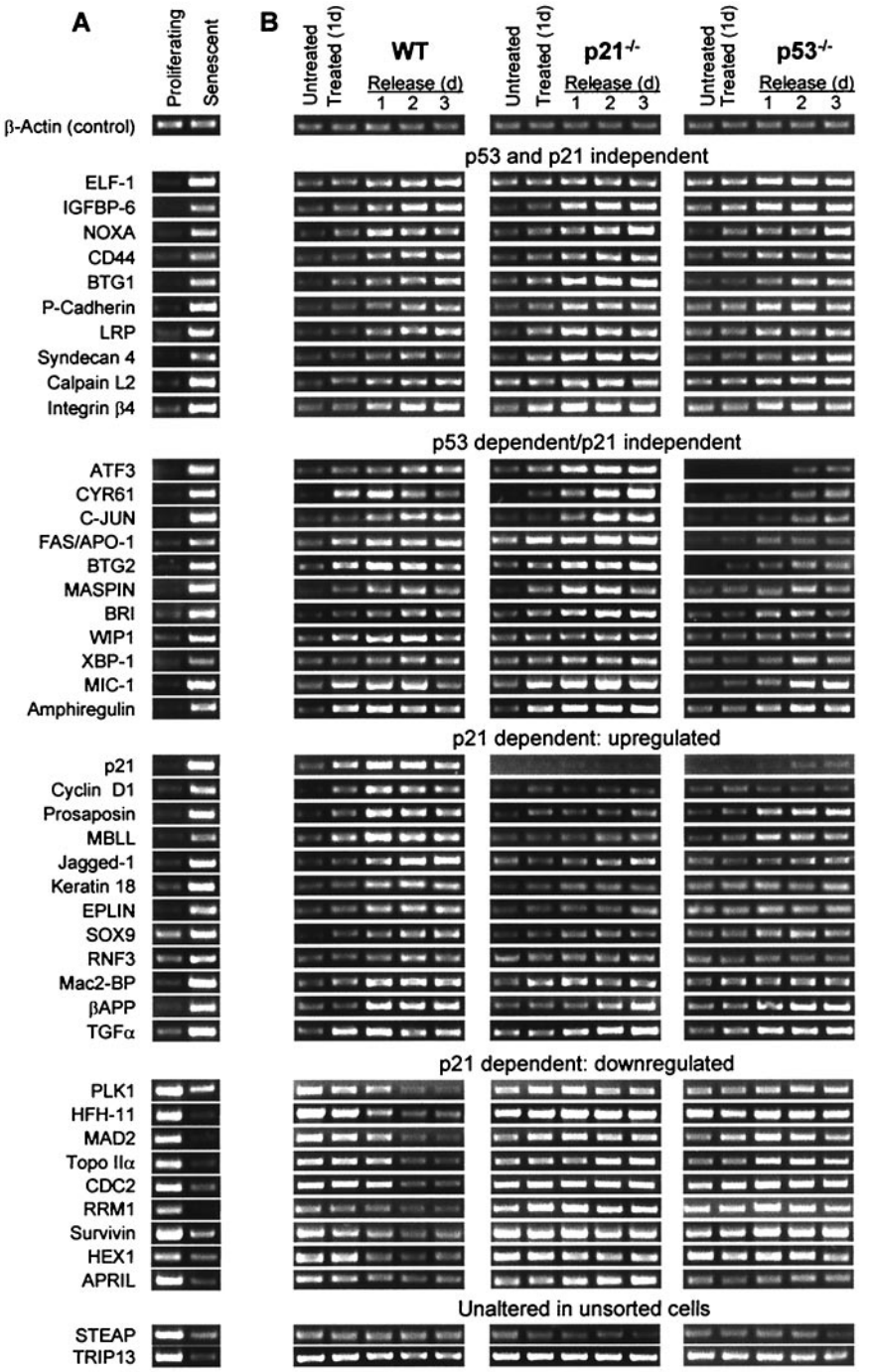

Fig. 2. RT-PCR analysis of changes in the expression of the indicated senescence-associated genes. β-actin was used as a normalization standard. (*A*) Gene expression in proliferating (PKH2^{lo}) and senescent (PKH2^{hi}) populations of HCT116 cells, separated 9 days after doxorubicin treatment. (*B*) Gene expression in the unsorted populations of wild-type, p21$-/-$, and p53$-/-$ HCT116 cells, before and after 24-hr treatment with 200 nM doxorubicin, and on the indicated days after release from the drug. Genes were designated as p53- or p21-dependent if changes in their expression became detectable at a later day or were less pronounced in the p53$-/-$ or p21$-/-$ lines than in the wild-type cells.

wild-type, p53$-/-$ (14), and p21$-/-$ (15) HCT116 cell lines demonstrated that p21 induction in this system strongly depends on p53 (Fig. 3*B*). Both p53 and p21 proteins are maintained at elevated levels in senescent cells isolated 9 days after release from the drug (Fig. 3*A*). In contrast to p21, however, p53 is up-regulated only at the protein level. In addition to sustained p21 induction, senescent cells strongly overexpress many other growth inhibitors, including several known or putative tumor suppressor genes. Some of these genes encode intracellular growth-inhibitory proteins, including tumor suppressor *BTG1* and its homolog *BTG2*, putative tumor suppressor *EPLIN*, and WIP1 phosphatase. Senescent HCT116 cells also overexpress several secreted growth inhibitors, including MIC-1 (pTGF-β), insulin-like growth factor binding protein-6 (IGFBP-6), serine protease inhibitor Maspin (a tumor suppressor down-regulated in advanced breast cancers), and amphiregulin (an epidermal growth factor-related factor that inhibits proliferation of several carcinoma cell lines while promoting the growth of normal epithelial cells). These findings suggest that drug-induced growth arrest of tumor cells is maintained by a set of apparently redundant intracellular and paracrine factors.

Differences in gene expression between senescent and proliferating populations of drug-treated HCT116 cells parallel the differences between normal and cancerous epithelial cells. In addition to the above-listed tumor suppressors, senescent HCT116 cells induce several other genes that are down-

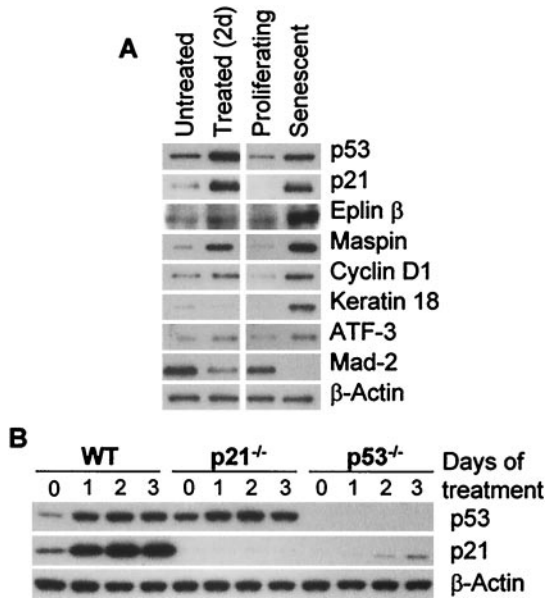

Fig. 3. Immunoblotting analysis of changes in p53 and the indicated protein products of genes that show altered expression in drug-induced senescence. β-actin was used as a normalization standard. (*A*) Immunoblotting of wild-type HCT116 cells that were either untreated, treated for 2 days with 200 nM doxorubicin, or sorted into proliferating ($PKH2^{lo}$) and senescent ($PKH2^{hi}$) cell populations 9 days after doxorubicin treatment. (*B*) p53 dependence of p21 induction in doxorubicin-treated HCT116 cells. Immunoblotting analysis of the wild-type, p21−/−, and p53−/− HCT116 cell lines treated with doxorubicin for the indicated number of days.

regulated in cancers relative to normal epithelial cells (*MIC-1*, P-cadherin, desmoplakin, desmoyokin, and neurosin). On the other hand, senescent cells down-regulate not only multiple genes involved in cell proliferation but also some other genes that have oncogenic activity (*RHAMM* and *TLS/FUS*) or show tumor-specific expression (*STEAP*). Another sign of putative "normalization" of senescent cells is the up-regulation of six members of the keratin gene family. The strongest induction in this group was observed for keratins 8 and 18, a keratin pair with antiapoptotic activity (16). Senescent HCT116 cells show no evidence of apoptosis, even though they up-regulate two proapoptotic genes, *APO-1/Fas* and *NOXA*.

Induction of Genes with Paracrine Growth-Promoting and Tissue-Reorganizing Activities. Aside from the growth inhibitors, senescent HCT116 cells show increased expression of genes for secreted mitogenic, antiapoptotic, and angiogenic factors, such as extracellular matrix (ECM) proteins Cyr61 and prosaposin, and transforming growth factor (TGF)-α. Induction of such genes and the corresponding paracrine activities, which promote tumor cell growth *in vitro* and *in vivo*, have been associated with replicative senescence (3) in normal cells and with p21 induction in tumor cells (17). Senescent cells also up-regulate several proteases (kallikrein-7, calpain L2, neurosin, and urokinase-type plasminogen activator) that may potentially contribute to metastatic growth. Several other genes induced in senescent cells are involved in cell adhesion and cell–cell contact (e.g., P-cadherin, Mac2-binding protein, and desmoplakin). Other induced genes encode ECM receptors, including several integrins and syndecan-4 (ryudocan), involved in angiogenesis. Some other transmembrane proteins induced in senescent cells are growth-regulatory proteins CD44 and Jagged-1, Alzheimer's β-amyloid precursor protein (βAPP), and another amyloid precursor, BRI, associated with an Alzheimer's-like disease. Altogether, secreted factors, ECM proteins, ECM receptors, and other integral membrane proteins make up 33 of 68 genes with known functions that are induced in senescent HCT116 cells (in contrast, only 2 of 64 down-regulated genes with known function belong to this category).

Parallels with Replicative Senescence and Organism Aging. The pattern of gene expression in drug-induced senescence of HCT116 cells shows many similarities to the senescence of normal cells. Some of the general properties of senescent cells (other than terminal growth arrest), are resistance to apoptosis, increased cell adhesion associated with overproduction of ECM components, and secretion of proteases, protease inhibitors, and mitogenic factors (3). Genes involved in all of these phenomena are amply represented among those that are up-regulated in senescent tumor cells. In contrast to normal cells, however, senescent HCT116 cells do not up-regulate p16 or tumor suppressor *PML* associated with RAS-induced accelerated senescence (18). Changes in gene expression associated with drug-induced senescence also show parallels with organism aging. Some of the proteins that are induced in the senescent HCT116 colon carcinoma cells, such as βAPP and prosaposin, show age-dependent expression. Remarkably, Maspin, CD44, and Cyclin D1 were reported to be up-regulated specifically in the colonic epithelium of aging animals (19). In addition, eight genes down-regulated in senescent HCT116 cells also showed decreased expression in actively growing fibroblast cultures from old people relative to similar cultures from young people, whereas two induced genes (*MIC-1* and desmoplakin) were up-regulated in cultures from older individuals (20). It seems, therefore, that the process of drug-induced senescence in tumor cells is related to both replicative senescence and organism aging.

Altered Expression of Transcription Factors in Senescent Cells. Genes for several known or putative transcription factors and cofactors show altered regulation in the senescent HCT116 cells. One of the down-regulated transcription factors is winged helix protein HFH-11 (Trident), a positive regulator of DNA replication, specifically expressed in cycling cells (21). Several up-regulated transcription factors are related to the AP-1 family, which mediates cellular responses to various mitogenic signals, interferons, and different forms of stress (22). These include c-Jun and two other basic leucine zipper proteins, XBP-1 (structurally related to c-Jun) and ATF3, which dimerizes with c-Jun. Sustained up-regulation of ATF3 mRNA and protein in senescent cells is surprising, because induction of this stress-responsive factor is usually transient (over hours), due to the ability of ATF3 to inhibit its own transcription (23). Another induced transcription factor is ELF-1, a member of the Ets family of helix–loop–helix proteins that are known to interact functionally, and possibly physically, with AP-1 (22).

Senescence-Associated Changes in Gene Expression Overlap with the Effects of p53 and p21. Many of the genes that show altered expression in senescent HCT116 cells have shown similar changes after overexpression of p53 (9 down-regulated and 11 up-regulated genes) or p21 (46 down-regulated and 7 up-regulated genes) (see Tables 1 and 2). p53 acts as a direct transcriptional activator of many genes (including p21) and indirectly regulates a group of genes that do not have p53-binding sites in their promoters (24, 25). A prominent class of p53-induced genes encodes secreted growth-inhibitory factors, providing paracrine antiproliferative activity (24). In contrast to p53, p21 is not a transcriptional regulator *per se*, but it interacts with a broad network of transcription factors, cofactors, and mediators of signal transduction (26). Overexpression of p21 in fibrosarcoma cells results in down-regulation of multiple cell proliferation genes and up-regulation of many ECM components

and secreted mitogenic and antiapoptotic factors, providing the corresponding activities in conditioned media of p21-induced cells (17). A known mechanism for transcription activation by p21 is based on its ability to stimulate p300/CBP transcription cofactors (27). HCT116 cells, however, express an apparently dominant mutant form of p300 (28), which may explain why senescent HCT116 cells up-regulate a relatively small number of p21-inducible genes.

Effects of p53 and p21 Knockouts on Drug-Induced Changes in the Expression of Senescence-Associated Genes. To elucidate the roles of p53 and p21 in the observed changes in gene expression, we have analyzed the expression of senescence-associated genes after doxorubicin treatment of wild-type, p21−/−, and p53−/− HCT116 cells. RNA samples were isolated before the addition of the drug, immediately after 1-day treatment with doxorubicin and on 3 consecutive days after the removal of the drug. Expression of 33 genes that were up-regulated and 11 genes down-regulated in senescent cells was analyzed by RT-PCR (Fig. 2*B*). This analysis showed that all of the tested genes were expressed in the untreated wild-type cells at levels similar to those in the proliferating fraction of doxorubicin-treated cells. Senescence-associated changes in the expression of most of these genes became detectable in the total population of wild-type HCT116 cells after 1-day doxorubicin treatment or 1 day after release from the drug. This early response made it possible to evaluate the effects of p21 and p53 knockouts on total populations of doxorubicin-treated cells, without having to purify the small senescent fractions of p21−/− and p53−/− cell lines.

Approximately one-third of the genes that are up-regulated in senescent cells showed almost indistinguishable response among the wild-type, p21−/−, and p53−/− cell lines, indicating that the induction of these genes does not involve p53 or p21 (Fig. 2*B*). These genes include tumor suppressor *BTG1* and secreted growth inhibitor IGFBP-6. Surprisingly, one of the genes that shows no p53 dependence is *NOXA*, although it is known to be inducible by p53. The remaining two-thirds of the up-regulated genes showed diminished or delayed induction in p53−/− cells. About one-half of the latter genes was unaffected by p21 knockout. This group includes transcription factors of the AP-1 family, CYR61, and several intracellular (BTG2 and WIP1) and secreted growth inhibitors (Maspin, MIC-1, and amphiregulin). None of these genes, however, completely depend on p53 for their induction, and all of them are induced in p53−/− cells 2 days after release from the drug. Almost all senescence-associated growth inhibitors (except for p21 and EPLIN) are eventually induced in p53−/− cells to a level comparable to the wild-type cell line (Fig. 2*B*). These results provide an explanation for the diminished but still substantial induction of senescence-like growth arrest in p53−/− cells after doxorubicin treatment (8).

The final group of the induced genes shows much weaker changes in p21−/− than in the wild-type cells (Fig. 2*B*), indicating that regulation of these genes is mediated through p21. Because p21 induction in doxorubicin-treated HCT116 cells is p53-dependent, such genes also show diminished induction in p53−/− cells. The strongest p21 dependence among the tested genes is found for Cyclin D1. None of p21-dependent genes produces secreted growth inhibitors, but two of them encode secreted mitogenic/antiapoptotic proteins (prosaposin and TGF-α). Most of the genes that are down-regulated in senescent HCT116 cells are known to be inhibited by p21 (17). In agreement with this observation, such genes show decreased expression after doxorubicin treatment only in the wild-type but not in p21−/− or p53−/− cell lines (Fig. 2*B*). Together with the genes that show p21-dependent induction, 20 of 31 tested genes that are affected by p53 knockout (excluding p21) are also affected to the same or greater degree by the knockout of p21. Therefore, p21, which until recently was not known to play a role in the regulation of gene expression, seems to be a major mediator of the corresponding effects of p53.

Implications for Cancer Therapy. Induction of senescence-like growth arrest was shown to be a prominent response to different anticancer agents *in vitro* and in animal models, but no studies have yet investigated the induction of accelerated senescence in patients' tumors. Nevertheless, there is clinical evidence that prolonged cytostatic arrest in some cases may be the primary mode of tumor response to radiation therapy. In particular, complete regression of prostate cancers was reported to take more than 1 year in some patients (29), and regression of desmoid tumors took up to 2 years (30) after radiation treatment. This slow course of tumor disappearance seems most consistent with radiation-induced senescence.

Detection of the senescence response in clinical cancers requires diagnostic markers for senescent cells. The most common senescence marker, SA-β-gal, has two obvious disadvantages: it represents an enzymatic activity that is preserved only in frozen samples and for a limited period and it is not mechanistically related to growth arrest of senescent cells. We have now identified a number of up-regulated genes that are functionally related to growth arrest, such as p21, *EPLIN*, *BTG1*, *BTG2*, *WIP1*, Maspin, *MIC-1*, IGFBP-6, and amphiregulin. The products of these genes are expressed in senescent tumor cells at a much higher level than in untreated cells or in drug-treated proliferating cells (Figs. 2*A* and 3*A*), and these proteins may therefore provide sensitive diagnostic markers. Induction of these senescence-associated growth inhibitors is not limited to doxorubicin-treated HCT116 cells analyzed in the present study. For example, EPLIN, a growth-inhibitory protein that was down-regulated in 20 of 21 carcinoma cell lines relative to normal epithelial tissues (31), is strongly induced in MCF-7 breast carcinoma cells by treatment with retinoids, under the conditions that produce senescence-like growth arrest (32). Retinoid treatment was also shown to induce a secreted growth inhibitor IGFBP-6 (33). Most of the other genes have been shown to be induced by DNA damage in a variety of other tumor-derived cell lines. These include *BTG1* (34), *BTG2* (35), *WIP1* (36), Maspin (37), and *MIC-1* (24). Further analysis should indicate the utility of senescence-associated growth inhibitors as markers of treatment response in human cancer.

Elucidation of molecular changes associated with treatment-induced senescence also has potential therapeutic implications. Permanently arresting tumor cell growth through the induction of accelerated senescence seems an attractive treatment approach, because this response to drug treatment can be elicited even under the conditions of minimal cytotoxicity (1). Our finding that drug-induced senescence is associated with concerted induction of multiple antiproliferative genes (some of which also inhibit the growth of neighboring cells) suggests the existence of common regulatory pathways activating such genes. Importantly, most of the growth-inhibitory genes are also induced by doxorubicin treatment in p53-deficient cells. Agents that can be developed to stimulate the induction of senescence-associated growth-inhibitory genes are likely therefore to be efficacious against tumors with or without functional p53.

The obverse side of drug-induced senescence, however, is the induction of genes associated with pathological conditions (such as Alzheimer's disease), as well as proteases and mitogenic, antiapoptotic, and angiogenic secreted factors. Expression of such genes by senescent cells may have potentially adverse effects in the short term (growth stimulation of nonsenescent tumor cells) and in the long term (increased likelihood of *de novo* carcinogenesis and age-related diseases). A linkage between cell senescence and carcinogenesis *in vivo* has been suggested in a recent study by Paradis *et al.* (38), who found that SA-β-gal expression in normal human liver was strongly correlated with

the development of hepatocellular carcinoma. We have shown that p21 induction up-regulates many disease-associated genes and induces paracrine antiapoptotic and mitogenic activities (17). In the present study, p21 knockout was found to decrease or delay the induction of such genes as prosaposin, TGF-α, and Alzheimer's βAPP. These observations suggest that p21-stimulated regulatory pathways may be largely responsible for the expression of disease-associated genes in senescent cells.

Induction of disease-promoting genes, however, is not invariably linked to the senescent phenotype. In particular, cDNA microarray analysis of MCF-7 cells treated with *all-trans* retinoic acid (RA) under senescence-inducing conditions revealed induction of several growth-inhibitory genes but not of any genes with known mitogenic, antiapoptotic or pathogenic functions (32). Remarkably, RA-treated MCF-7 cells (in contrast to cells treated with DNA-damaging agents) showed no increase in p21 expression. These findings indicate that it is possible to induce senescence-associated growth inhibitors without concurrent induction of tumor-promoting and disease-associated factors. In the present study, we have identified specific genes associated with the beneficial and detrimental aspects of drug-induced senescence in tumor cells and obtained further evidence for the role of p21 in the negative aspects of senescence. These findings suggest potential strategies for developing agents that would promote terminal growth arrest of tumor cells or diminish senescence-associated side effects of cancer therapy.

We thank B. Vogelstein for the wild-type, p21$-/-$, and p53$-/-$ HCT116 cell lines and D. Chang for anti-EPLIN Ab. This work was supported by National Institutes of Health Grants R01 CA89636, R01 CA62099, and R37 CA40333 (to I.B.R.), training grant T32 DK0773909 (to M.E.S.), and by American Cancer Society (Illinois Division) Grant 00-35 (to B.D.C.).

1. Chang, B. D., Broude, E. V., Dokmanovic, M., Zhu, H., Ruth, A., Xuan, Y., Kandel, E. S., Lausch, E., Christov, K. & Roninson, I. B. (1999) *Cancer Res.* **59,** 3761–3767.
2. Goodwin, E. C., Yang, E., Lee, C. J., Lee, H. W., DiMaio, D. & Hwang, E. S. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 10978–10983.
3. Campisi, J. (2000) *In Vivo (Athens, Greece)* **14,** 183–188.
4. Di Leonardo, A., Linke, S. P., Clarkin, K. & Wahl, G. M. (1994) *Genes Dev.* **8,** 2540–2551.
5. Robles, S. J. & Adami, G. R. (1998) *Oncogene* **16,** 1113–1123.
6. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. (1997) *Cell* **88,** 593–602.
7. Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D. & Barrett, J. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13742–13747.
8. Chang, B. D., Xuan, Y., Broude, E. V., Zhu, H., Schott, B., Fang, J. & Roninson, I. B. (1999) *Oncogene* **18,** 4808–4818.
9. Jordan, M. A., Wendell, K., Gardiner, S., Derry, W. B., Copp, H. & Wilson, L. (1996) *Cancer Res.* **56,** 816–825.
10. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I. & Pereira-Smith, O. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9363–9367.
11. Noonan, K. E., Beck, C., Holzmayer, T. A., Chin, J. E., Wunder, J. S., Andrulis, I. L., Gazdar, A. F., Willman, C. L., Griffith, B., Von Hoff, D. D., *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7160–7164.
12. Myohanen, S. K., Baylin, S. B. & Herman, J. G. (1998) *Cancer Res.* **58,** 591–593.
13. Horan, P. K. & Slezak, S. E. (1989) *Nature (London)* **340,** 167–168.
14. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W. & Vogelstein, B. (1998) *Science* **282,** 1497–1501.
15. Waldman, T., Kinzler, K. W. & Vogelstein, B. (1995) *Cancer Res.* **55,** 5187–5190.
16. Caulin, C., Ware, C. F., Magin, T. M. & Oshima, R. G. (2000) *J. Cell Biol.* **149,** 17–22.
17. Chang, B. D., Watanabe, K., Broude, E. V., Fang, J., Poole, J. C., Kalinichenko, T. V. & Roninson, I. B. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4291–4296.
18. Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., *et al.* (2000) *Nature (London)* **406,** 207–210.
19. Lee, H., Greeley, G. H. & Englander, E. W. (2001) *Mech. Ageing Dev.* **122,** 355–371.
20. Ly, D. H., Lockhart, D. J., Lerner, R. A. & Schultz, P. G. (2000) *Science* **287,** 2486–2492.
21. Ye, H., Holterman, A. X., Yoo, K. W., Franks, R. R. & Costa, R. H. (1999) *Mol. Cell Biol.* **19,** 8570–8580.
22. Wisdom, R. (1999) *Exp. Cell Res.* **253,** 180–185.
23. Wolfgang, C. D., Liang, G., Okamoto, Y., Allen, A. E. & Hai, T. (2000) *J. Biol. Chem.* **275,** 16865–16870.
24. Komarova, E. A., Diatchenko, L., Rokhlin, O. W., Hill, J. E., Wang, Z. J., Krivokrysenko, V. I., Feinstein, E. & Gudkov, A. V. (1998) *Oncogene* **17,** 1089–1096.
25. Zhao, H. & Xu, Y. H. (1999) *Cell Res.* **9,** 51–59.
26. Dotto, G. P. (2000) *Biochim. Biophys. Acta* **1471,** M43–M56.
27. Snowden, A. W., Anderson, L. A., Webster, G. A. & Perkins, N. D. (2000) *Mol. Cell Biol.* **20,** 2676–2686.
28. Gayther, S. A., Batley, S. J., Linger, L., Bannister, A., Thorpe, K., Chin, S. F., Daigo, Y., Russell, P., Wilson, A., Sowter, H. M., *et al.* (2000) *Nat. Genet.* **24,** 300–303.
29. Cox, J. D. & Kline, R. W. (1983) *Int. J. Radiat. Oncol. Biol. Phys.* **9,** 299–303.
30. Bataini, J. P., Belloir, C., Mazabraud, A., Pilleron, J. P., Cartigny, A., Jaulerry, C. & Ghossein, N. A. (1988) *Am. J. Surg.* **155,** 754–760.
31. Maul, R. S. & Chang, D. D. (1999) *Oncogene* **18,** 7838–7841.
32. Dokmanovic, M., Chang, B. D. & Roninson, I. B. (2002) *Cancer Biol. Ther.* **1,** 16–19.
33. Dailly, Y. P., Zhou, Y., Linkhart, T. A., Baylink, D. J. & Strong, D. D. (2001) *Biochim. Biophys. Acta* **1518,** 145–151.
34. Cortes, U., Moyret-Lalle, C., Falette, N., Duriez, C., Ghissassi, F. E., Barnas, C., Morel, A. P., Hainaut, P., Magaud, J. P. & Puisieux, A. (2000) *Mol. Carcinog.* **27,** 57–64.
35. Fletcher, B. S., Lim, R. W., Varnum, B. C., Kujubu, D. A., Koski, R. A. & Herschman, H. R. (1991) *J. Biol. Chem.* **266,** 14511–14518.
36. Fiscella, M., Zhang, H., Fan, S., Sakaguchi, K., Shen, S., Mercer, W. E., Vande Woude, G. F., O'Connor, P. M. & Appella, E. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6048–6053.
37. Zou, Z., Gao, C., Nagaich, A. K., Connell, T., Saito, S., Moul, J. W., Seth, P., Appella, E. & Srivastava, S. (2000) *J. Biol. Chem.* **275,** 6051–6054.
38. Paradis, V., Youssef, N., Dargere, D., Ba, N., Bonvoust, F., Deschatrette, J. & Bedossa, P. (2001) *Hum. Pathol.* **32,** 327–332.